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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) Mucin glycoproteins are highly expressed by many tumors, reduce normal cell-cell and cell-extracellular matrix adhesion and protect cancer cells from attack by the immune system. Mucin expression not only increases, but also changes from a restricted pattern of apical expression to a general distribution over the entire cell surface. In this regard, conversion of prostate epithelium from a highly-organized, growth-controlled phenotype to a highly proliferative, metastatic phenotype is associated with loss of cell polarity. Very few studies been performed on mucin expression by prostate cancer cells. MUC1 is a large molecular weight, type I transmembrane mucin glycoprotein expressed by normal and malignant prostate epithelium. High level cell surface expression, reported immunosuppressive activities of its released ectodomain, and antiadhesive properties all contribute to this mucin's ability to protect and promote tumor cell growth and survival. Recent observations using human breast cancer cell lines indicate that MUC1 can associate with the intracellular signal transducing molecules, β -catenin and GRB-2. Recent studies from the PI's lab demonstrate that cytokines, including interferon- γ , markedly stimulate MUC1 gene expression. Primary prostate tumors are often found in the vicinity of cytokine producing cells, and commonly metastasize to bone marrow, a rich source of these same cytokines.					
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Introduction

MUC1 is a large, polymorphic mucin expressed on the surfaces of many normal and malignant epithelia (for review see references 1 and 2). Like other mucins, MUC1 is believed to provide protection to mucosal surfaces from both microbial and enzymatic attack as well as lubricate these cell surfaces. Over the last few years, it has become appreciated that MUC1 functions are more diverse. MUC1 is antiadhesive and inhibits cell-cell and cell-extracellular matrix interactions in both normal, e.g., embryo implantation, and pathological, e.g., cancer cells, contexts. In the case of cancer cells, MUC1 is often highly overexpressed and its antiadhesive properties may promote cell detachment from primary tumor sites as well as protection of the tumor cells from cell-mediated lysis. MUC1 expression is strongly regulated by steroid hormones in breast and uterine tissues *in vivo* and by proinflammatory cytokines *in vitro*.

The large externally-disposed portions of MUC1 (ectodomains) are released from normal and tumor cells where they can both absorb antibodies generated to tumor-specific MUC1 glycoforms as well as suppress immune cell function. Moreover, MUC1 has a transmembrane/cytoplasmic tail region that is highly conserved across species suggesting a conserved function. Recent studies indicate that the MUC1 cytoplasmic tail interacts with important signal transducing molecules, e.g., β -catenin and Grb2, and may participate in signal transduction events (3, 4). In spite of the number of studies of MUC1 expression and function in other systems, very little is known about MUC1 in the context of prostate cancer beyond that it is expressed in normal prostate epithelia and primary tumors. The proposed studies examine the impact of cytokines and androgens on MUC1 expression and function in androgen-sensitive and insensitive prostate cancer cell lines as well as normal prostate epithelia both at the level of gene expression and interactions with signal transducing proteins. A MUC1 gene knockdown approach will be used to disrupt MUC1 interactions with intracellular signal transducing molecules to determine the impact this has on prostate cancer cell growth.

Body

Our research accomplishments are detailed below, following the organization of the original proposal. MUC1 expression observed in most of the prostate cancer cell lines chosen for study has proven to be very low and not stimulated by either cytokines or testosterone. We extended these studies by examining MUC1 expression in tissue sections of primary prostate tumors provided by collaborators at Emory University. We have added studies of normal prostate epithelia to determine if regulation of MUC1 expression is fundamentally different between normal and transformed cells in this tissue.

Task I – To examine interferon- γ and androgen modulation of MUC1 gene expression

We have used both Western and Northern blotting approaches to examine MUC1 expression in primary cultures of normal human prostate epithelial cells (PrEC), LnCaP, C4-2, C4-2B, PC-3 as well as DU145 cell lines cultured with and without the presence of interferon- γ , TNF- α , dihydrotestosterone (DHT). PrEC cells displayed very little MUC1

by Western blotting; however, this expression could be stimulated by the addition of cytokines (interferon- γ plus TNF α [I+T]), but not DHT (Fig. 1). Thus, normal prostatic epithelia require cytokine stimulation to produce MUC1. PC-3 cells responded similarly, except that interferon- γ alone was sufficient to drive MUC1 expression. In contrast, DU145 cells constitutively expressed MUC1 and this expression was not further stimulated by cytokines or DHT (Fig. 2). MUC1 was not detected in LnCaP, C4-2 or C4-2B cells under any condition (Fig. 3 shows data for C4-2B cells). In light of our preliminary observations that MUC1 was readily detectable by immunohistochemistry in normal prostate epithelia and primary tumors, we considered that loss of expression in the cell lines might be due to: 1) the *in vitro* culture conditions; 2) loss of MUC1 expression in metastatic cells (all three cell lines were derived from metastases) or; 3) the requirement for combinations of cytokines and/or hormones for high level MUC1 expression in prostate cancer cells. We conducted additional studies of MUC1 expression in sections of over 300 primary prostate tumors in a human prostate tumor tissue microarray provided by collaborators at Emory University (a few representative photos are shown in Fig. 4 below). These studies revealed highly variable expression of MUC1. In some cases, strong staining consistent with cell surface expression was detected (left hand panels), while in other cases intracellular staining was evident (right hand panels); however, in most

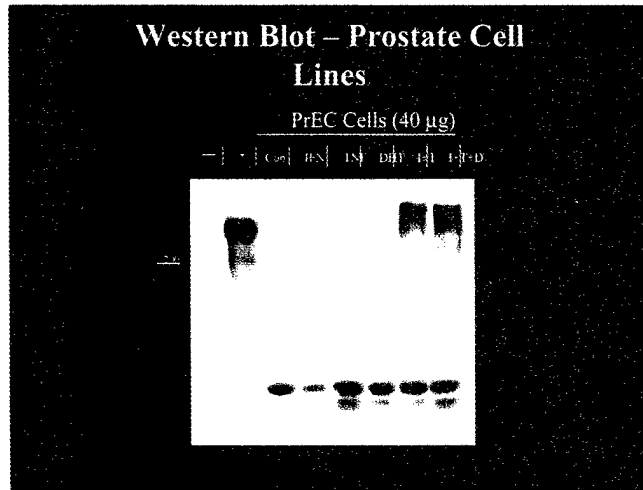


Figure 1

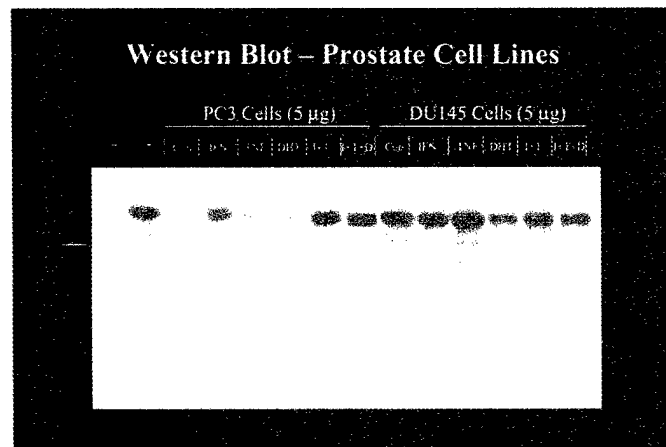


Figure 2

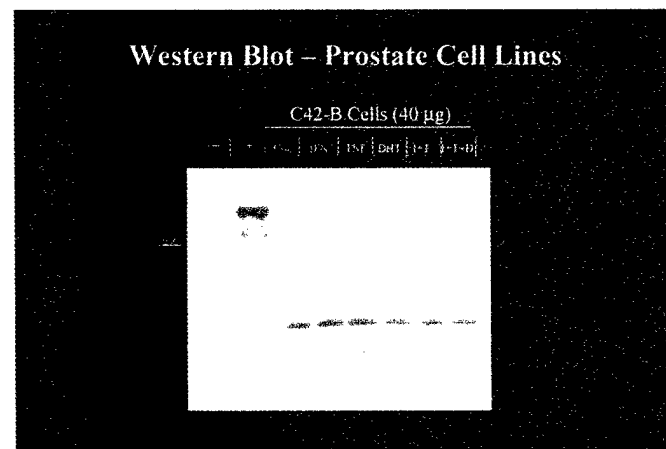


Figure 3

cases (57%) little or no staining was evident. Similar results were obtained using antibodies directed at either the ectodomain or cytoplasmic tail domains. In addition, in collaboration with Dr. Robert Sikes (Univ. of Delaware) we found that injection of either LnCaP or LnCaP-derived C42-B cells into mouse bone marrow failed to stimulate MUC1 expression in these cells (data not shown). Thus, even the complex mixture of factors present in bone marrow is insufficient to stimulate MUC1 expression in these androgen-responsive and non-responsive tumor cell lines. Additional work will determine if the MUC1-positive primary tumors correspond to a particular tumor grade as determined by an experienced pathologist through our collaboration at Emory University.

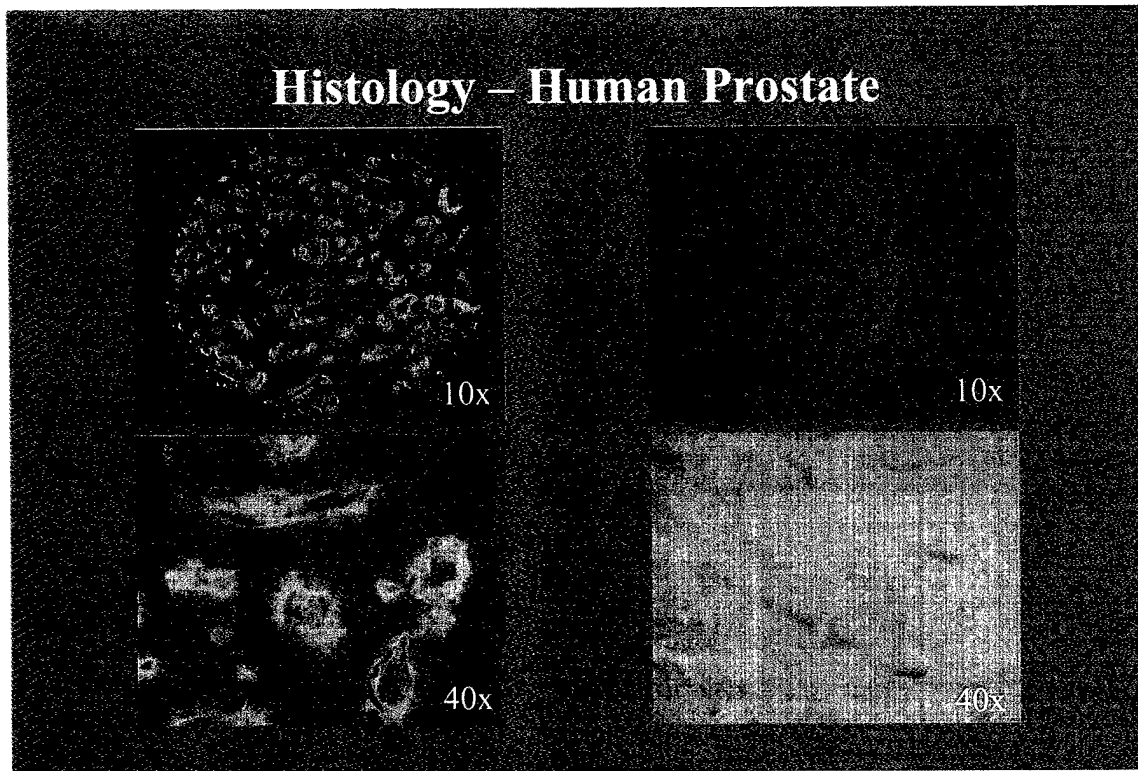


Figure 4

Task II – To define parameters by which interferon- γ or androgen modulate MUC1 association with β -catenin and GRB-2

These studies were originally planned to be initiated in months 16-22. In light of the inability to stimulate MUC1 expression in most primary prostate tumors and prostate cancer cell lines we have abandoned these studies.

Task III – To test effects of disruption of formation of MUC1 complexes with β -catenin and GRB-2 on prostate cancer cell growth in vitro

These studies were originally planned to be initiated in months 22-36. In light of the inability to stimulate MUC1 expression in most primary prostate tumors and prostate cancer cell lines we have abandoned these studies.

Key Research Accomplishments

- 1) Determination that neither interferon- γ , TNF- α nor DHT alone or in combination stimulates MUC1 expression in 4 out of 5 prostate cancer cell lines.
- 2) Determination that combined treatment of interferon- γ and TNF- α stimulates MUC1 expression in normal prostate epithelia and PC-3 cells.
- 3) Determination that MUC1 and MUC1SEC mRNA are expressed by cytokine-treated normal prostate epithelia.
- 4) Determination that cell-associated MUC1 in normal prostate epithelia is in the form of an SDS-dissociable complex of the transmembrane/cytoplasmic tail with the ectodomain.
- 5) Determination that MUC1 is not highly expressed in a large fraction (>50%) of primary prostate tumors

Reportable Outcomes

Parts of this work have been published as aspects of two manuscripts. A third manuscript summarizing most of the work described in Aim 1 is being prepared.

Conclusions

It appears that neither normal prostate epithelia nor prostate cancer cell lines express high levels of MUC1 in their basal states. Normal prostate epithelia will do so when stimulated with combinations of cytokines (interferon- γ and TNF- α) shown to greatly stimulate MUC1 expression in other cellular contexts. These observations suggest that synergistic actions of cytokines, and perhaps androgen, are required to augment MUC1 production in prostate cancer cells as originally proposed. In spite of this, these cytokines alone or in combination with DHT do not stimulate MUC1 expression in 4 out of 5 cell lines tested. Moreover, neither androgen-responsive or non-responsive prostate cancer cell lines express MUC1 when injected into bone marrow in mice. Finally, we have found that while MUC1 is readily detectable in some primary prostate tumors, it is not in most. In light of these results, it does not appear to be fruitful to use MUC1 as a target of prostate cancer diagnosis or therapy.

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Formation of MUC1 metabolic complex is conserved in tumor-derived and normal epithelial cells

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Abstract

MUC1 is abundantly expressed at the surface of epithelial cells in many tissues and their carcinomas. In humans, genetic polymorphism and differential splicing produce isoforms that may contribute to MUC1 participation in protection of the cell surface, modulation of cell–cell interactions, signaling, and metastasis. Biosynthetic and processing studies in tumor-derived cell lines indicate that cell surface MUC1 consists of a non-covalently associated heterodimer of separate cytoplasmic tail and extracellular domains. This heterodimer results from a single precursor proteolytically cleaved intracellularly. To determine whether similar processing of this isoform occurs in normal epithelial cells, we have examined cell-associated MUC1 and MUC1 released into medium by normal human uterine, mammary, and prostate epithelial cells. Cell-associated MUC1/REP was extracted as an SDS labile complex which was resistant to dissociation by boiling, urea, sulfhydryl reduction, peroxide, high salt, or low pH and was present in all normal cells examined. Addition of various proteolytic inhibitors during extraction had no effect on the proportion of this complex detected. MUC1 released into the medium was not associated with a particulate fraction (100,000g insoluble) and lacked the cytoplasmic tail. MUC1/REP and the MUC1 isoform lacking the transmembrane/cytoplasmic tail region, MUC1/SEC, mRNA were detected in all normal cells examined indicating that both shed and secreted MUC1 are likely to contribute to soluble forms found in culture media. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: MUC1; Mucin; Epithelia; Processing; Human; Uterus; Prostate

The extensively glycosylated ectodomains of transmembrane mucins protect the apical surface of normal glandular epithelium and generally create a surface non-adhesive to a variety of environmental pathogens and toxins as well as apposing epithelial cells [1–3]. Maintenance of this barrier function, as well as other functions attributed to transmembrane mucins, would be dependent upon regulation of mucin expression at the levels of transcription and translation, proper post-translational processing, and ultimately, regulation of ectodomain release. In normal cells, mechanisms by which these events are accomplished may be common for transmembrane mucins, regardless of species or tissue in which the individual mucins are expressed. Nonetheless, most studies of these events have utilized tumor derived or transformed cell lines in which altered

transcriptional regulation [4–6], alternative splicing [7–9], aberrant glycosylation [10,11], and altered cellular distribution can occur [12,13]. Of all the transmembrane mucins identified so far, the most extensively examined is MUC1.

The polymorphic mucin MUC1 is expressed by most normal glandular epithelia and is found as both cell-associated and soluble forms (reviewed in [14,15]). Allelic variations in the number of tandem repeats in the extracellular domain of human MUC1 and alternative mRNA splicing contribute to the array of MUC1 forms detected in various systems. The predicted amino acid sequence describes a type I transmembrane protein possessing an extended extracellular domain composed mostly of 20 amino acid repeats varying in number with the individual alleles, a transmembrane domain, and a cytoplasmic tail of 69 amino acids [16–18]. Additional cell-associated forms, MUC1/Y [19,20] and MUC1/Z [21], identical to the full length molecule, but lacking the tandem repeats, have been detected in malignant cells.

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Soluble forms include the ectodomain of full length MUC1, termed MUC1/REP and a secreted form resulting from an alternative splicing event in which a stop codon is introduced into the sequence preceding the transmembrane and cytoplasmic domains, MUC1/SEC [18,22].

In certain tumor cells, full length MUC1 protein arrives at the cell surface as a heterodimeric complex, the result of an intracellular proteolytic cleavage of a single precursor protein shortly after translation in the endoplasmic reticulum [23]. The two cleavage products remain stably associated throughout intracellular processing and delivery to the cell surface. Although the association appears to be non-covalent, it survives multiple rounds of recycling from the cell surface to the Golgi for additional glycosylation and return to the surface [24]. Initial studies using recombinant MUC1 identified two potential cleavage sites within an 18 amino acid sequence located between 71 and 53 amino acid N-terminal to the transmembrane domain [23]. A subsequent study utilizing an epitope-tagged MUC1 has determined that one of the two Phe–Arg dipeptides is the intracellular cleavage site [25]. Furthermore, the cleavage event proved to be independent of host cell and uninfluenced by heavily O-glycosylated tandem repeat domains [25].

The soluble forms of MUC1 may be distinguished from each other by a unique amino acid sequence which lies at C-terminal on MUC1/SEC [22]. Both forms lack the transmembrane domain and cytoplasmic tail. While the tailless MUC1/SEC is clearly the product of alternative splicing, the more abundant form, MUC1/REP, is released from full length MUC1 under conditions in which alternative splicing could not occur [26]. The mechanism of release remains to be determined. Two possibilities have been suggested: dissociation of the complex formed as a result of metabolic processing [25] or a second cleavage (shedding) event [23]. Importantly, all of these studies have utilized cell lines derived from adenocarcinomas or transformed cell lines. Consequently, concern exists over whether formation of the heterodimeric MUC1 complex is a feature of tumor cells and not necessarily preserved in their normal counterparts. Therefore we have examined the processing of endogenous MUC1 in normal epithelial cells originating from several human tissues. These studies demonstrate that intracellular cleavage and formation of the heterodimeric complex are common to both normal and cancer cells and cell lines.

Materials and methods

Cell lines and culture conditions. The human uterine epithelial cell line, HES [27], was cultured in 24-well plates on matrigel (Collaborative Research) coated wells in high glucose Dulbecco's

modified eagle's medium (Gibco BRL) supplemented with 10% (v/v) charcoal stripped fetal bovine serum (Hyclone), 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.1 mM sodium pyruvate. Normal human mammary epithelial cells, HMEC, and normal human prostate epithelial cells, HPrE, were cultured on untreated 24-well plates in medium specified for each cell type by the supplier (Clonetics). MUC1 expression was induced by treatment with 200 U/ml interferon γ (IF γ) and 25 ng/ml tumor necrosis factor α (TNF α) for 48 h. The human mammary carcinoma cell line, T47D, was obtained from the American Tissue Culture Collection and cultured in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin.

Preparation of cell lysates. Unless otherwise specified, cell lysates were prepared from confluent cultures by addition of 0.5% (v/v) Nonidet P-40 in phosphate-buffered saline (PBS) containing 1 mM ethylenediaminetetraacetic acid and a protease inhibitor mixture at a final dilution of 1:100 (v/v). The protease inhibitor mixture was composed of 10 mg/ml chymostatin, 1 mg/ml pepstatin, 1 mg/ml leupeptin, 10 mg/ml benzamide, 2 mg/ml antipain, and 7.6 trypsin-inhibiting U/ml aprotinin in 0.9% (w/v) NaCl and 0.9% (v/v) benzyl alcohol. Lysis buffer was added at 98 °C. After 5 min at room temperature, the cell residue was scraped into the lysis buffer and the lysate was centrifuged for 15 min at 4 °C at 10,000g.

Treatment of cell lysates. Cell lysates were exposed to 6 M urea, 5% (v/v) β -mercaptoethanol, or 1% (w/v) sodium dodecyl sulfate (SDS) at room temperature for 30 min. Urea or β -mercaptoethanol was removed by passing the samples over dextran desalting columns (Pierce) equilibrated with 0.5% (v/v) Nonidet P-40 (NP-40) in PBS. The sample containing SDS was diluted to 0.1% (w/v) SDS with 0.5% (v/v) NP-40 in PBS. To determine the effect of low pH on the MUC1 metabolic complex, lysate was brought to pH 5.0 by the addition of HCl, incubated for 1.5 h at room temperature, and brought back to pH 7.4 by addition of NaOH. To determine the effect of high salt, lysate was made 2 M NaCl by the addition of 4 M NaCl in PBS, incubated for 1.5 h at room temperature, and diluted to 0.15 M NaCl with 0.5% (v/v) NP-40. To test the effect of elevated temperature, lysate was boiled for 10 min and chilled to room temperature. Lysate was incubated for 2 h with 100 µM hydrogen peroxide at room temperature, followed by removal of peroxide by passage over a dextran desalting column. Metalloprotease inhibitor Illomostat (GM6001, Chemicon) was dissolved in dimethyl sulfoxide and added to lysis buffer at a concentration of 10 µg/ml. Furin inhibitor ([28]; CalBiochem) was dissolved in methanol and added to cell culture medium and lysis buffer at a concentration of 25 µM. After all treatments, the lysate was divided equally prior to immunoprecipitation.

Preparation of total cell extracts and released protein. For preparation of total cell extracts, confluent cultures were solubilized by addition of 0.05 M Tris, pH 7.0, 8 M urea, 1.0% (w/v) SDS, 1.0% (v/v) β -mercaptoethanol, and 0.01% (w/v) phenylmethylsulfonyl fluoride at room temperature. For collection of released/secreted proteins, cultures were rinsed once with serum-free medium and cultured for 24 h in serum-free medium. Conditioned medium was centrifuged at 4 °C for 10 min at 10,000g and used directly for immunoprecipitation or concentrated by precipitation at 4 °C with 10% (w/v) trichloroacetic acid (2 µl fetal bovine serum was added per 250 µl medium as carrier). Precipitates were rinsed once with acetone, air dried, and dissolved in equal volumes of sample extraction buffer (above) and Laemmli's sample buffer [29] containing 20% (v/v) glycerol. Total cell extracts were precipitated as described, without carrier.

Vectorial biotinylation of MUC1 at the cell surface. Confluent cultures of HES were aseptically rinsed with ice-cold PBS containing 2 mM CaCl₂ and 2 mM MgCl₂ (PBS-CM) and incubated at 4 °C for 30 min in the dark with 10 mM sodium metaperiodate in 0.1 M sodium acetate, pH 5.0. Periodate oxidation was terminated by addition of cold PBS-CM containing 200 mM sucrose for 5 min followed by two

rinses with Hank's balanced salt solution (HBSS). Cells were then incubated at 4°C for 2 h in the dark with 100 µg/ml biotin hydrazide (Pierce) in HBSS. The reaction was terminated by rinsing three times with ice-cold HBSS. Prewarmed serum-free medium was added and the cultures were processed immediately or returned to the incubator for 24 h at 37°C. Control cultures were rinsed with cold PBS and maintained at 4°C in PBS for the same period of time but were not subjected to the biotinylation protocol. In this protocol, the O-linked glycans are biotinylated rather than the protein core.

Immunoprecipitation and Western blot analysis. Lysate (125 µl) or medium (500 µl), representing one half of sample obtained from a confluent well, was incubated by constant rotary agitation overnight at 4°C with rabbit polyclonal antibody CT-1 [30,31] at the ratio of 1:60, serum to medium or 1:15, serum to lysate. Mouse monoclonal antibody 214D4 [32] was added at the ratio of 1:25, hybridoma medium to medium or 1:6, hybridoma medium to lysate. Antigen-antibody complexes were incubated for 8 h at 4°C with constant agitation after addition of 50 µl of a 50% (v/v) slurry of protein G-Sepharose (Sigma) that had been previously blocked with fetal bovine serum. The pelleted resin was washed twice with 500 µl of 0.5% (v/v) NP-40 in PBS and twice with PBS. Resin pellets were extracted by boiling 2 min in 50 µl sample extraction buffer (above) and 50 µl Laemmli's sample buffer, followed by centrifugation. Samples (25 µl) were separated on 10% (w/v) SDS-polyacrylamide gels [33], transferred to nitrocellulose in 100 mM Tris base, 100 mM glycine, pH 8.3, and blocked for 4 h at 4°C in 3% (w/v) BSA (fraction V, Sigma), 0.1% (v/v) Tween 20 in PBS. Primary antibody 214D4 was used at a dilution of 1:10,000 in blocking buffer. After an overnight incubation at 4°C, the blots were rinsed three times for 5 min in 0.5% (v/v) Tween 20 in PBS. Secondary antibody, HRP-conjugated donkey anti-mouse IgG (Jackson Immunologicals), diluted 1:200,000 in blocking buffer was added for 2 h at 4°C. Blots were rinsed three times 5 min in 0.5% Tween 20 in PBS. Antibody was visualized by ECL reagents applied according to manufacturer's directions (Pierce).

RNA isolation and RT-PCR analysis. Total RNA was isolated from confluent cultures using an RNeasy kit and QIA-shredder columns according to manufacturer's directions (Qiagen). Genomic sequences were removed by deoxyribonuclease treatment (DNA-free Kit, Ambion) and a mock treated sample of each was performed in parallel. Total RNA (1 µg) was reverse transcribed in a final volume of 20 µl reaction mixture using random hexamers and kit components at the recommended concentrations (Gene Amp RNA PCR Kit, Applied Biosystems). The reaction mixture was maintained at room temperature for 10 min, 15 min at 42°C, 5 min at 99°C, and 5 min at 4°C. A negative control lacking template was included in each reverse transcription and subsequent amplification experiment. In addition, a duplicate sample containing template but lacking reverse transcriptase was included. Samples for PCR were prepared by addition of 10 µl of RT reaction to 40 µl of a PCR mixture containing kit components at the recommended concentrations and primers (0.2 µM each). Primers for MUC1/REP corresponded to bases 729–748 (forward primer: TGCATCAGGCTCAGCTTCTA) and bases 1257–1276 (reverse primer: GAAATGGCACATCACTCACG); product size was 548 bp. Primers for MUC1/SEC corresponded to bases 729–748 (forward primer: TGCATCAGGCTCAGCTTCTA) and bases 1068–1087 (reverse primer: GGAAGGAAAGGCCGATACTC); product size was 359 bp. Primers for human ribosomal protein L19 were: forward primer-CTGAAGGTGAGGGGAATGTG and reverse primer-GGATAAAGTCTTGATGATCTC; product size was 239 bp. After an initial incubation for 2 min at 95°C, samples were amplified for 35 cycles of 1 min at 95°C, 1 min at 58°C, and 1 min at 72°C for MUC1/REP and MUC1/SEC or 25 cycles of 1 min at 95°C, 1 min at 58°C, and 1 min at 72°C for L19 with a final period of 5 min at 72°C. PCR products were resolved on 1.2 % (w/v) agarose gels in 40 mM Tris acetate 2 mM ethylenediaminetetraacetic acid, pH 8.5.

Results and discussion

Cell-associated MUC1 exists as an SDS-labile complex in normal epithelial cells

Using the human breast cancer cell line T47D, one of the cell lines in which the initial observations on the metabolic cleavage and complex formation of MUC1 were made [23], we first confirmed the nature of the MUC1 complex and examined conditions that might affect its formation and/or dissociation. In parallel, the cell line HES, a cell line derived from normal proliferative phase human uterine epithelium [27], was examined. Cell-associated MUC1 was extracted with NP-40 in the presence or absence of β-mercaptoethanol, urea or SDS. If the complex remained intact, all conditions would yield similar amounts of MUC1 immunoprecipitated with the cytoplasmic tail-directed antibody (CT-1). If, however, the complex was disrupted by one or more conditions, this would be reflected in the diminished ectodomain signal when immunoprecipitated with antibody recognizing the cytoplasmic tail. As was the case for cell-associated MUC1 extracted from ZR-75-1 breast carcinoma cells [23], MUC1 extracted from HES could be dissociated by exposure to SDS, but not urea or β-mercaptoethanol (Fig. 1a). Exposure of cell-associated MUC1 to high salt, low pH, or peroxide also failed to dissociate the complex (Figs. 1b and c). Cell-associated MUC1 extracted from T47D breast cancer cells displayed a similar sensitivity to SDS (data not shown). Utilizing the susceptibility of the complex to SDS disruption, two other normal epithelial cells were examined for the presence of MUC1 metabolic complex. In this case, the cells were induced to produce sufficient MUC1 for analysis by combined treatment with interferon γ and TNFα (E. Lagow and D.D. Carson, manuscript submitted). Cell-associated MUC1 in both normal human mammary epithelial cells (HMEC) and normal human prostate epithelial cells (HPrEs) exhibited a similar susceptibility to SDS (Fig. 1d). Thus, cell-associated MUC1 in normal cells appears to undergo a similar metabolic cleavage event and form an SDS-dissociable complex as reported for tumor derived cell lines.

The use of SDS disruption to demonstrate the presence of the metabolic complex raised the question of what portion of endogenous full length MUC1 was subjected to cleavage. In some early experiments, exposure to SDS led to a reduction, but not complete disappearance of detectable MUC1 ectodomain precipitable by CT1. This could have resulted either from a partial cleavage of MUC1 during metabolic processing or an incomplete dissociation of the preformed complex during exposure to SDS. Extending the period of exposure to SDS to 1 h produced a uniform complete disruption of the metabolic complex as determined by

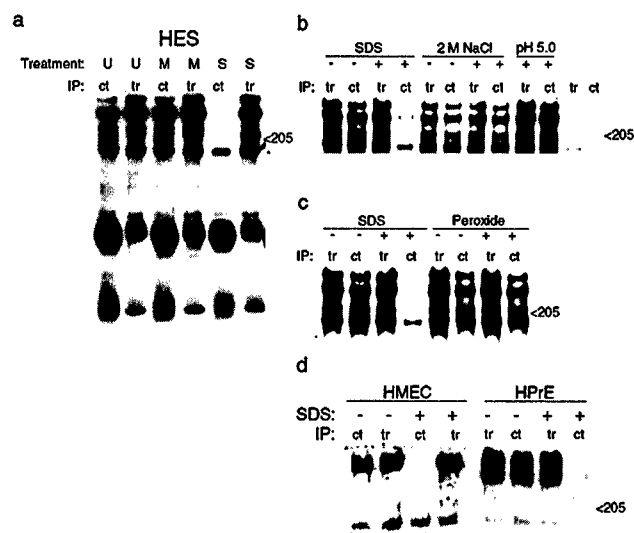


Fig. 1. Cell-associated MUC1 expressed by normal human epithelial cells exists as an SDS labile complex. (a) Prior to immunoprecipitation, lysates of HES were treated with either 6M urea (U), 5% (v/v) β -mercaptoethanol (M), or 1% (w/v) SDS (S). Treated lysates were split equally and immunoprecipitated with either anti-C terminal peptide, CT-1 (ct), or anti-tandem repeat, 214D4 (tr). The Western blot was probed by antibody 214D4. Bands detected below 205 kDa are due to components of the IgG used for immunoprecipitation (*). (b) Prior to immunoprecipitation, lysates of HES were treated with either 1% (w/v) SDS, 2M NaCl, or pH 5. Treated lysates were split equally and immunoprecipitated as described in panel A. The last two lanes contain no lysate, only antibody. The Western blot was probed by antibody 214D4. (c) Prior to immunoprecipitation, lysates of HES were treated with either 1% (w/v) SDS or 100 μ M hydrogen peroxide. Treated lysates were split and immunoprecipitated as described in panel A. The Western blot was probed by antibody 214D4. (d) Prior to immunoprecipitation, lysates of HMEC or HPrE were treated with (+) or without (–) 1% (w/v) SDS. Treated lysates were split equally and immunoprecipitated as described in panel A. The Western blot was probed by antibody 214D4.

the lack of detectable MUC1 ectodomain precipitable by CT-1 and this condition was used in all subsequent experiments. This result agrees with the observation of Parry et al. [25] that almost no uncleaved MUC1 could be detected in whole cell lysates of several different tumor cell lines originating from various tissues. Therefore, metabolic cleavage and heterocomplex formation appear to be an intrinsic feature of full length MUC1 processing in normal epithelial cells as well and is independent of the tissue source.

The extended processing time involved in extraction, SDS exposure, and immunoprecipitation of samples led us to consider the possibility that a portion of the “metabolic cleavage” may occur after disruption of the cell and exposure of MUC1 to solubilized proteases that would not otherwise have access to MUC1 during its transit through or residence at the surface of the intact cell. Two lines of evidence provided in the studies of others indicate that this is probably not the case. MUC1 translated in vitro or within a cell context and extracted

under similar conditions demonstrated a progressive degree of cleavage dependent upon the period of metabolic processing [23]. Although these experiments were performed with a truncated form of MUC1 containing only one tandem repeat, it has been determined in a subsequent study that neither the number of tandem repeats nor the degree of glycosylation of the tandem repeats affects the site or extent of metabolic cleavage [25]. To exclude the possibility of post-extraction cleavage, we evaluated the effect of various conditions of extraction on the proportion of complex detected. Cells were extracted by the addition of boiling 0.5% Nonidet P40 containing 1 mM EDTA, and a cocktail of serine protease inhibitors for 5 min, 0.5% Nonidet P40 containing no inhibitors at 4°C for 1 h or 0.5% Nonidet P40 containing 1 mM EDTA and a cocktail of serine protease inhibitors at 4°C for 1 h. Neither elevated temperature nor inclusion of a proteolytic inhibitor cocktail reduced the subsequent proportion of SDS labile complex in the resulting lysate (data not shown). Inclusion of a broad spectrum metalloproteinase inhibitor, Illomostat [34], in addition to the protection afforded by 1 mM EDTA, did not affect the proportion of SDS-labile complex present in the resulting lysates (Fig. 2a). Pretreatment during culture and inclusion of a furin inhibitor during lysis also did not affect the proportion of SDS-labile complex (Fig. 2b). Contrary to the results of Ligtenberg et al. [23], we could find no evidence that exposure to elevated temperature resulted in the dissociation of the metabolic complex. Neither lysis at elevated temperature nor boiling the lysate after extraction (Fig. 2a) resulted in dissociation of the complex unless SDS was present.

Normal epithelial cells release a tailless, soluble form of MUC1

The ultimate fate of the MUC1 metabolic complex upon arrival at the cell surface remained to be determined in normal cell lines. Release of the ectodomain as a soluble form rather than lysosomal degradation appears to be the fate of the majority of MUC1 arriving at the surface of tumor cells. In tumor cell lines all surface-associated MUC1 molecules were constitutively internalized and recycled to the surface until release [24]. Transport to the lysosomes was considered to be of minor importance. As noted earlier, the two soluble forms of MUC1 released by tumor cell lines lack the cytoplasmic tail. We confirmed that the forms of MUC1 released into the medium by normal human epithelial cells were not membrane-associated (Fig. 3) and lacked the cytoplasmic domain (Fig. 4). These experiments further demonstrated that the MUC1 released to the medium from normal cells could not be the product of cell lysis which would release membrane fragments containing MUC1 metabolic complex. If this were the

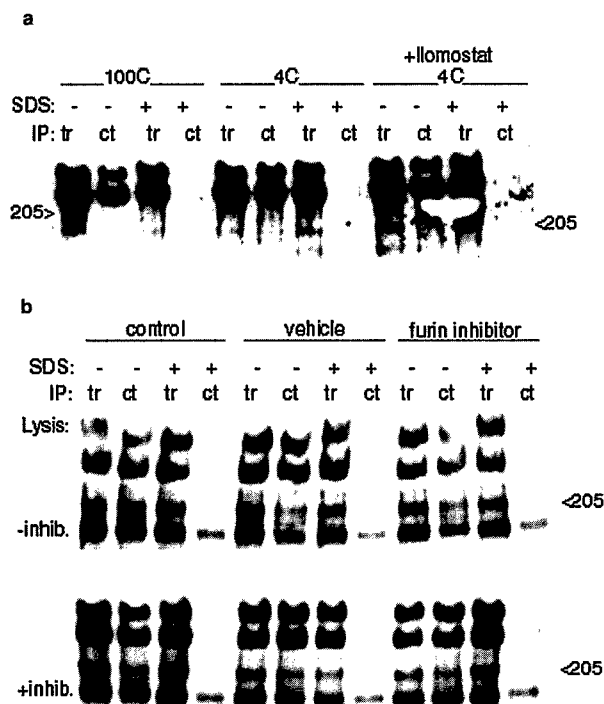


Fig. 2. MUC1 metabolic complex expressed by normal human epithelial cells does not dissociate at 100°C and is not further proteolytically cleaved during lysis. (a) HES were lysed in the standard lysis buffer with or without 100 μg/ml Illomostat for 1 h at 4°C or lysis buffer was added at 98°C, followed by 5 min at room temperature and 10 min at 100°C before centrifugation. The treated lysates were split equally and immunoprecipitated with anti-C terminal peptide, CT-1 (ct) or anti-tandem repeat, 214D4 (tr). The Western blot was probed by antibody 214D4. (b) HES cultures were incubated for 24 h with no addition (control), vehicle, or 25 μM furin inhibitor prior to extraction. Lysates were prepared in standard lysis buffer containing vehicle (upper set) or 25 μM furin inhibitor (lower set). Lysates were split equally and immunoprecipitated as described in panel A. The Western blot was probed by antibody 214D4.

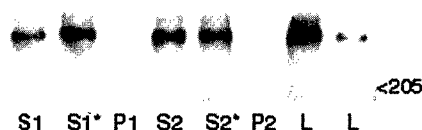


Fig. 3. MUC1 released into medium by HES is not associated with particulates. Serum free medium conditioned by HES for 24 h was centrifuged for 10 min at 10,000g. Two 2 ml samples were further centrifuged for 1 h at 100,000g. The upper 1 ml of each supernate (S1, S2) and the lower 1 ml (S1*, S2*) were removed and the bottom of the tubes were extracted with sample extraction buffer (P1, P2). All were TCA precipitated as described in Materials and methods. A 1 ml and 0.5 ml sample not subjected to 100,000g centrifugation (L) were included for comparison. Precipitates were resolved by SDS-PAGE and the Western blot was probed by antibody 214D4.

case then CT-1 antibody would have immunoprecipitated the MUC1 ectodomain. However, these experiments do not distinguish between the two possible soluble forms of MUC1. Although production of the splice variant MUC1/SEC has been detected in

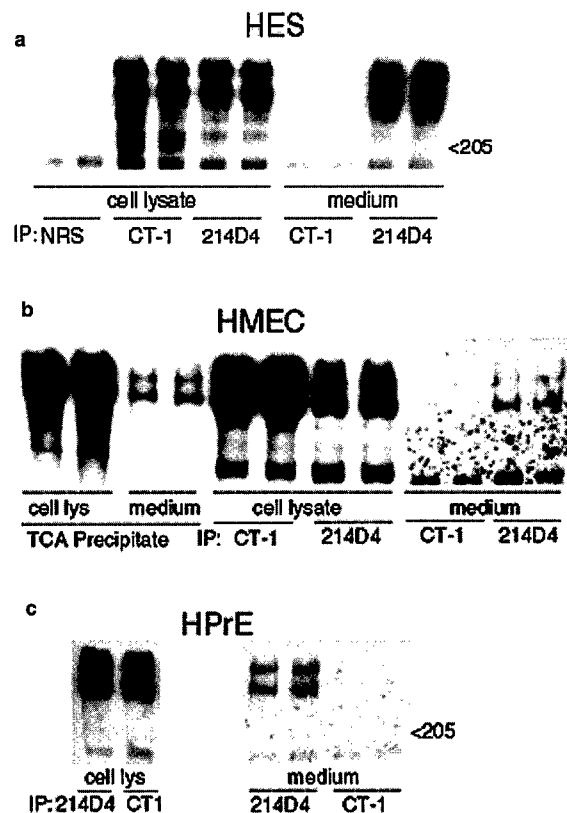


Fig. 4. MUC1 released into medium by normal human epithelial cells lacks the cytoplasmic tail. (a) Serum free medium conditioned by HES for 24 h or cell lysate was immunoprecipitated with normal rabbit serum (NRS), anti-C terminal peptide (CT-1) or anti-tandem repeat (214D4) in duplicate. The Western blot was probed by antibody 214D4. (b) Serum free medium conditioned by HMEC for 48 h or cell lysate was immunoprecipitated as in panel A or TCA precipitated in duplicate as described in Materials and methods. The Western blot was probed by antibody 214D4. (c) Serum free medium conditioned by HPrE for 48 h or cell lysate was immunoprecipitated as in panel A. The Western blot was probed by antibody 214D4.

secretions of normal fallopian tube and uterus of a lower primate [35], its production by normal human cells has not been examined. If MUC1/SEC could not be detected in normal human cells, the threefold possibilities for the origin of the tailless MUC1 ectodomain in secretions from a variety of normal human tissues would be reduced to two: it is either generated from the dissociation of the MUC1 metabolic complex or it results from an additional proteolytic cleavage step. However, mRNA for MUC1/SEC was detected by RT-PCR analysis of total RNA extracted from HES, HMEC, or HPrE (Fig. 5). Thus, a portion of soluble MUC1 released by these cells could be due to secretion of MUC1/SEC. Nonetheless, the majority of soluble MUC1 must arise from the predominant form of MUC1 expressed by normal cells, MUC1/REP. Under conditions in which alternative splicing was not possible, transformed mouse mammary cells transfected with full length human

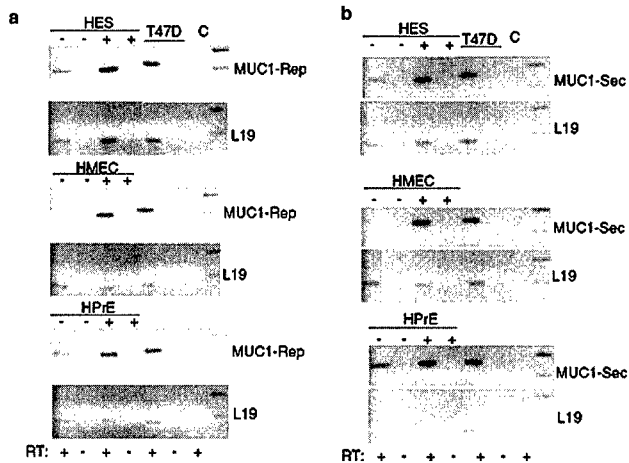


Fig. 5. Normal human epithelial cells express mRNA for both MUC1/REP and MUC1/SEC. Total RNA extracted from HES, HMEC, HPrE, or T47D was analyzed by RT-PCR for the expression of mRNA encoding MUC1/REP (panel a), MUC1/SEC (panel b), and ribosomal protein L19. HES were either cultured in medium containing 10% (v/v) serum (+) or withdrawn from serum for 48 h (–). As indicated at the top of each series, HMEC and HPrE were cultured either with (+) or without (–) interferon γ and TNF α for 48 h as described in “Materials and methods.” As indicated at the bottom of each series, reverse transcription (RT) was performed on 1 μ g total RNA in the presence (+) or absence (–) of reverse transcriptase. Control (C) contained no template.

MUC1 released a tailless form of MUC1 into the medium [26]. As demonstrated in Fig. 6, MUC1 biotinylated at the cell surface appears as a tailless form released to the medium within 24 h. During the same time period, cell-associated, biotinylated MUC1, precipitable by both CT-1 and antibody 214D4, disappeared (data not shown). MUC1/SEC would not be expected to be retained sufficiently long at the cell surface to become biotinylated nor would it be immunoprecipitated by the CT-1 antibody.

The remaining question is the pathway of release: dissociation of the metabolic complex or an additional cleavage step. A simple dissociation is unlikely for a number of reasons, first of which is the apparent stability of the metabolic complex. The complex must remain associated not only through the steps of metabolic processing from the endoplasmic reticulum to delivery at the cell surface, but it must also be sustained through multiple rounds of recycling from the cell surface to the golgi for additional glycosylation (sialylation) with return to the surface [24]. The results of the present study further demonstrate the stability of the complex when exposed to conditions that might be encountered at the surface or during recycling. Secondly, the failure to detect an additional cleavage fragment, i.e., an N-terminus distinct from that of the metabolic cleavage site [25], does not exclude the possibility of a second cleavage event. Those studies were performed on immunoprecipitated cell-associated metabolic complex. If a second

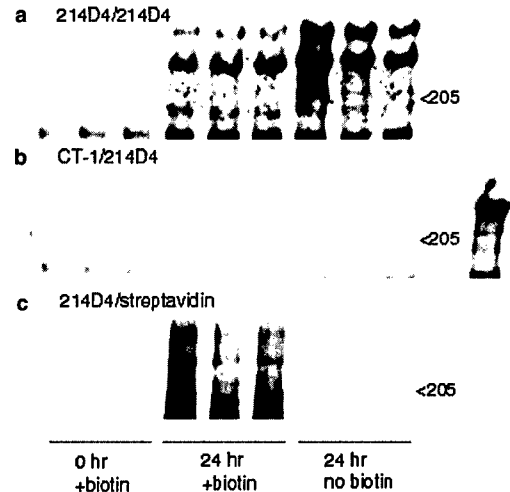


Fig. 6. MUC1 vectorially biotinylated at the cell surface is released into the medium lacking the cytoplasmic tail. HES were biotinylated at the cell surface as described in “Materials and methods.” Control cultures were rinsed and maintained at 4 °C, but were not biotinylated. Serum free medium was added and collected immediately after biotinylation (0 h) or after 24 h incubation at 37 °C. Equal amounts of medium were immunoprecipitated with anti-tandem repeat (214D4; panels a and c) or anti-C terminal peptide (CT-1; panel b). Western blots of triplicate individual samples were probed by antibody 214D4 (panels a and b) or HRP-streptavidin (panel c). The immunoprecipitating antibody/Western blot probe is indicated above each panel. The sample to the right of the middle panel is the last control from the top panel included as a positive control for the Western blot.

cleavage event had occurred, the residual membrane-associated fragment with a potentially different amino terminus would not have been included in the immunoprecipitates produced by the antibody to the FLAG sequence incorporated into the ectodomain. Finally, if no complex was formed, no release of ectodomain would be expected if the mechanism was simple dissociation; however, cells transfected with a mutant form of full length MUC1 lacking the metabolic cleavage site were reported to release as much MUC1 as those expressing the wild type form [23]. Since no metabolic complex was formed, release of the ectodomain had to have occurred by another mechanism.

These results indicate that processing of the full length MUC1 core protein is similar in both normal and tumor cells, regardless of differences in glycosylation or the production of isoforms. The identity of the protease responsible for the metabolic cleavage, thought to be a member of the kallikrein family [23,25], remains to be determined, as does the mechanism by which the ectodomain is ultimately released from the metabolic complex. Comparison of amino acid sequences suggests that the metabolic cleavage of MUC1 is conserved across species [25]. Cleavage and formation of a metabolic complex as an initial step of metabolic processing are predicted to occur for MUC3 and MUC12 [25] based on sequence homologies to the MUC1 cleavage

site, and is a feature of MUC4 metabolism [36]. Although the cleavage site sequence in MUC4 differs from that of the other transmembrane mucins, it is also conserved across species and complex formation occurs in both normal tissues and tumor cells [5]. However, metabolic cleavage may not prove to be a universal event in transmembrane mucin processing. Epiglycanin, a large transmembrane murine mucin for which the human homolog is yet to be identified, does not form a metabolic complex [37].

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Synergistic Stimulation of MUC1 Expression in Normal Breast Epithelia and Breast Cancer Cells by Interferon- γ and Tumor Necrosis Factor- α

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Abstract The MUC1 gene encodes a transmembrane mucin glycoprotein that is overexpressed in human breast cancers. Persistent stimulation by proinflammatory cytokines may contribute to increased MUC1 transcription by tumor cells. We demonstrate that MUC1 expression in T47D breast cancer cells and normal human mammary epithelial cells (HMEC) is enhanced by tumor necrosis factor- α (TNF- α) in the presence of interferon- γ (IFN- γ). MUC1 responsiveness to these cytokines was modest in T47D cells and robustly induced in HMEC. Transient transfection of T47D cells with mutant MUC1 promoter constructs revealed that a κ B site at –589/–580 and the STAT-binding element at –503/–495 and were required for cooperative stimulation by TNF- α and IFN- γ . Binding of NF κ B p65 to the MUC1 κ B site was induced by TNF- α treatment, as demonstrated by electrophoretic mobility shift assay. Specific mutation of the κ B site prevented binding of NF κ B p65 and blocked TNF- α stimulation of MUC1 promoter activity. Collectively, these studies demonstrate synergistic stimulation of MUC1 expression by TNF- α and IFN- γ that is mediated by independent actions of NF κ B p65 and STAT1 α upon κ B and STAT sites, respectively, in the MUC1 promoter. Strong induction of MUC1 expression by these proinflammatory cytokines is clearly evident in normal mammary epithelium. In contrast, breast tumor cells appear to override normal regulatory responses via as yet undefined *cis*-elements. *J. Cell. Biochem.* 86: 759–772, 2002.

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Key words: MUC1; transcriptional regulation; tumor necrosis factor- α ; interferon- γ ; nuclear factor kappa B; signal transducer and activator of transcription

The full-length product of the MUC1 gene is a large type I transmembrane mucin glycoprotein primarily expressed on the apical surfaces of many mammalian simple epithelial cells that line ducts or glands [as reviewed in Gendler and Spicer, 1995; Lagow et al., 1999; Hanisch and Muller, 2000], although MUC1 expression by nonepithelial cells including hematopoietic cells [Dent et al., 1999], T cells [Agrawal et al., 1998a], and male germ cells [Franke et al., 2001] also has been reported. Proposed functions for MUC1 include modulation of cell adhesion,

signal transduction, lubrication and hydration of epithelial surfaces, and protection of epithelial surfaces from infection [Gendler and Spicer, 1995; Lagow et al., 1999; Hanisch and Muller, 2000]. In human breast cancers, regulation of MUC1 expression differs between breast tumor cells and adjacent normal epithelial cells via aberrant glycosylation, cellular distribution, and mRNA splicing; however, the molecular control of any of these events is not well understood [Girling et al., 1989; Hilken et al., 1992; Zrihan-Licht et al., 1994; Brockhausen et al., 1995]. MUC1 is greatly overexpressed in many epithelial-derived cancers including those of breast, ovary, and pancreas [Gendler and Spicer, 1995]. Overexpression of MUC1 in human breast cancers is frequently due to increased transcription [Hareuveni et al., 1990], possibly as a result of chromosomal rearrangement or gene duplication [Gendler et al., 1990; Bieche and Lidereau, 1997; Waltz et al., 1998].

Normal expression of MUC1 is under the control of steroid hormones in the mammary

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gland, although these responses appear to be indirect [Parry et al., 1992; Zhou et al., 1998]. Expression of MUC1, a constituent of milk, in normal human and mouse mammary epithelial cells is low in virgin glands but increases from mid-pregnancy to lactation [Burchell et al., 1987; Parry et al., 1992]. The development of mice transgenic for human MUC1, antigenically distinct from the endogenous mouse homologue, has brought to light the genomic requirements for expression and regulation of MUC1 in both normal breast and mammary tumors. The smallest transgene utilized to date that displayed expected epithelial-specific expression and regulation of MUC1, including increased expression in mammary tumors, utilized 1.4 kb of 5'-flanking sequence upstream of MUC1 cDNA, implying that introns and 3'-flanking sequence do not participate in transcriptional control [Graham et al., 2001].

A consistent observation among several MUC1-expressing cancer cell lines is the requirement of sequences between -600 and -400 for maximal expression [Abe and Kufe, 1993; Kovarik et al., 1993]. The importance of this region is supported by the finding that the internal sequence -598/-485 enhanced activity of a heterologous promoter more than tenfold in MCF7 breast cancer cells [Abe and Kufe, 1993; Kovarik et al., 1993]. Located within this transcriptionally important region of the MUC1 promoter are a potential binding site for the transcription factor NF κ B (nuclear factor kappa B) at -589/-580, overlapping with a putative AP-3 (activator protein 3) element [Abe and Kufe, 1993], and a STAT (signal transducer and activator of transcription) binding element at -503/-495. MUC1 regulation by cytokines and peptide hormones that signal through NF κ B and STAT transcription factors is well-documented [Tran et al., 1988; Parry et al., 1992; Clark et al., 1994; Lagow and Carson, 1999; Grunberg et al., 2000; Lagow and Carson, 2000; Gaemers et al., 2001], and evidence suggests that persistent cytokine stimulation may contribute to the increased transcription of MUC1 observed very frequently in breast cancers. The STAT-binding element at -503/-495 was shown to mediate interferon- γ (IFN- γ) responsiveness of the MUC1 gene in a breast cancer cell line, T47D, and specific mutation of the site reduced MUC1 promoter activity, suggesting its involvement in constitutive and stimulated MUC1 expression in these cells [Lagow and

Carson, 1999; Gaemers et al., 2001]. However, nuclear extracts from unstimulated T47D cells did not react with the STAT-binding element, indicating that the high level of MUC1 transcription observed in these cells is not due to constitutive activation of this single *cis*-acting promoter element. Furthermore, IFN- γ stimulation of MUC1 promoter activity did not translate into increased MUC1 expression in T47D cells. Therefore, we examined normal mammary epithelial cells to determine whether the mechanism for IFN- γ stimulation of MUC1 expression is conserved or is specific to tumor cells. Importantly, several proinflammatory cytokines including IFN- γ and tumor necrosis factor- α (TNF- α) are produced by lymphocytes associated with mucin-expressing breast tumors [Vitolo et al., 1992, 1993]. Therefore, overexpression of MUC1 may be at least partially the result of a combination of stimulatory factors acting upon multiple regulatory elements. For this reason, we investigated the role of the potential κ B site at -589/-580, in the context of the STAT-binding element, in transcriptional regulation of MUC1 by TNF- α and IFN- γ .

METHODS

Cell Culture

T47D human breast ductal carcinoma cells (ATCC, Manassas, VA) were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin (Life Technologies, Gaithersburg, MD). Human mammary epithelial cells, or HMEC (Clonetics, San Diego, CA), were maintained in the recommended mammary epithelial growth medium (MEGM) containing 10 ng/ml recombinant human EGF, 5 μ g/ml insulin, 0.5 μ g/ml hydrocortisone, 50 μ g/ml gentamicin, 50 ng/ml amphotericin B, and 13 mg/ml bovine pituitary extract (Clonetics).

Generation of Mutant Promoter Constructs

A genomic fragment consisting of the 5' flanking sequence of the MUC1 gene from -1,406 to +33 was a generous gift from Dr. Sandra Gendler (Mayo Clinic, Scottsdale, AZ). The 1.4MUC construct used in these studies previously was made by cloning this fragment into pGL3basic luciferase reporter vector (Promega, Madison, WI). PCR was used to generate 5'

deletion mutants of 1.4MUC, and inserts consisting of MUC1 promoter fragments -604/+33 and -487/+33 were cloned into pGL3basic. The pGL3-TK plasmid was constructed by cloning the *Bgl*II/*Hind*III fragment of pRL-TK (Promega), containing the HSV-TK promoter, into pGL3basic. Single-stranded oligos (Sigma-Genosys, The Woodlands, TX) encompassing -518/-480 of MUC1 gene sequence were annealed and cloned into pGL3-TK for analysis of the STAT-binding site. Sequence of the STAT-binding site in the native insert: 5'-TTCCGGGAA-3'; in the mutated insert: 5'-ccCCGGGAA-3' (mutated nucleotides in lowercase). The -604/-468 MUC1 promoter segment, containing the STAT-binding site and a potential κ B site, was amplified by PCR and cloned into pGL3-TK. All positive clones were confirmed by sequencing.

Site-Directed Mutagenesis

Recombinant PCR, as described by Kovarik et al. [1993], was used to generate specific mutations in the potential κ B site at -589/-580 and in the STAT-binding site at -503/-495 in the MUC1 promoter using the 1.4MUC construct as a template. The native sequence of the potential κ B site in the sense strand is 5'-GGAAAGTCCG-3', and the mutated sequence is 5'-GGcccGTCCG-3'. The native sequence of the STAT-binding site is 5'-TTCCGGGAA-3', and the mutated sequence is 5'-ccCCGGGAA-3'. Recombinant products containing one of the above mutations were used to replace the native sequence in the 1.4MUC construct.

Transient Transfections and Reporter Assays

Transient transfections were performed using LipofectAMINE reagent (Life Technologies) in a 6-well plate format according to the manufacturer's instructions. Two micrograms of pGL3basic-based plasmid and 0.25 μ g of pRL-TK plasmid were used per well. For co-transfection of 1.4MUC with STAT1 plasmids, a total of 3.25 μ g DNA were used per well: 2 μ g of 1.4MUC, 0.25 μ g pRL-TK, and 1 μ g of expression plasmids for human STAT1 α , STAT1 β , or vector. STAT1 α and STAT1 β expression plasmids were gifts from Dr. Ulrike Schindler (Tularik, Inc., South San Francisco, CA). Following transfection, cells were given fresh medium with 5% (v/v) fetal bovine serum in the presence or absence of

the following: 200 U/ml recombinant human IFN- γ (Roche, Indianapolis, IN), 25 ng/ml recombinant human TNF- α (Roche), or 10 ng/ml recombinant human IL-1 β (Roche). Normal rabbit serum (NRS) was purchased from Calbiochem-Behring (LaJolla, CA), and polyclonal rabbit-anti-human IFN- γ was purchased from BioSource (Camarillo, CA). Luciferase assays were performed using the Dual-Luciferase Assay Kit (Promega) according to the manufacturer's instructions and a Dynex MLX Microplate Luminometer (Dynex Technologies, Chantilly, VA). Reporter activity was expressed as the ratio of firefly luciferase activity (pGL3-based plasmids) to *Renilla* luciferase activity (pRL-TK). Statistical analyses were performed using GraphPad InStat software (GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego California USA, www.graphpad.com), employing one-way ANOVA and the Tukey-Kramer multiple comparisons test.

Northern Blot Analysis

T47D cells were plated in 6-well plates and maintained as described until cells reached confluence. Confluent cells were serum-starved for 24 h prior to treatment. For treatment, T47D cells were cultured in medium containing 5% (v/v) fetal bovine serum \pm 200 U/ml IFN- γ and/or 25 ng/ml TNF- α . The same treatments were administered to HMEC in MEGM, but using 50 ng/ml TNF- α . Total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA), and Northern blot analysis was performed on total RNA (6 μ g/lane for T47D RNA and 10 μ g/lane for HMEC RNA) using the NorthernMax-Gly kit (Ambion, Austin, TX) according to the manufacturer's instructions. The cDNA probe used for detection of MUC1 mRNA was a 436-bp RT-PCR product that encompasses parts of exons 6 and 7, encoding the cytoplasmic tail. A cDNA probe for human 18S ribosomal RNA (ATCC) was used as a load control. Probes were non-isotopically labeled using the BrightStar Psoralen-Biotin kit (Ambion), and hybridization signals were detected using the BrightStar BioDetect kit (Ambion). Blots were exposed to X-ray film, and signal intensities were quantified using the Alpha Imager 1D-Multi function (Alpha Innotech, San Leandro, CA). Blots were stripped between probings with boiling 0.1% (w/v) SDS in water, then stored at 4°C.

Western Blot Analysis

Cell treatments and sample preparation. T47D cells were seeded into 24-well plates and maintained as described until cells reached confluence. Confluent cells were serum-starved for 24 h, then cultured in serum-free medium \pm 200 U/ml IFN- γ and/or 25 ng/ml TNF- α . The same treatments were administered to HMEC in MEGM. Cells were solubilized with sample extraction buffer [SEB; 0.05 M Tris pH 7, 8 M urea, 1% (w/v) SDS, 0.01% (v/v) phenylmethylsulfonylfluoride (PMSF), 1% (v/v) β -mercaptoethanol], and protein concentration was determined by the method of Lowry et al., [1951]. Culture supernatants were centrifuged briefly at 4°C, 10,000g, to pellet any cell debris. Supernatants were precipitated overnight at 4°C using 50 μ g of fetal bovine serum protein as carrier. Resulting pellets were rinsed with acetone and resuspended in 25 μ l SEB and 25 μ l Laemmli sample buffer (LSB, [Laemmli, 1970]). Forty percent of each sample of precipitated culture supernatant protein and 10 μ g (T47D) or 50 μ g (HMEC) of each sample of cell-associated protein were analyzed. Each sample of cell-associated protein was brought up to a final volume of 25 μ l with SEB and LSB at 1:1 (v/v). Before loading, samples were heated at 95°C for 2 min, and 1 μ l of bromophenol blue (1% v/v in water) was added to each for visual tracking.

SDS-PAGE and detection of MUC1 protein. Proteins were separated by SDS-PAGE using a 4.5% (w/v) Laemmli stacking gel [Laemmli, 1970] and a 10% (w/v) Porzio and Pearson resolving gel [Porzio and Pearson, 1977] under constant current. Separated proteins were transferred to Schleicher & Schuell Protran nitrocellulose (Intermountain Scientific, Kaysville, UT) at 4°C. Nitrocellulose blots were blocked at 4°C in phosphate-buffered saline plus 0.1% (v/v) Tween-20 (Sigma) containing 3% (w/v) bovine serum albumin (Sigma). Primary antibody 214D4 (kindly provided by Dr. John Hilkens of The Netherlands Cancer Institute, Amsterdam, The Netherlands), a mouse monoclonal specific for a tandem repeat epitope in the extracellular domain of MUC1 [Wesseling et al., 1995], was added directly to the blocking solution to a final dilution of 1:10,000. In a comparison of 214D4 with several other antibodies directed toward the MUC1 tandem repeats (BC3 [Xing et al., 1989], DF3

[Perey et al., 1992], HMFG1, HMFG2 [Taylor-Papadimitriou et al., 1981; Burchell et al., 1983], and SM3 [Burchell et al., 1987]), 214D4 reacted more strongly and consistently in Western blots than any other antibody in a variety of samples (J. Julian, unpublished observations). Therefore, the 214D4 antibody was used in routine analyses. Following overnight incubation at 4°C, blots were rinsed at room temperature three times, 5 min each, in phosphate-buffered saline plus 0.1% (v/v) Tween-20. For detection, blots were incubated for 2 h at 4°C with horseradish peroxidase-conjugated sheep-anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) at a final dilution of 1:200,000 in blocking solution. After three 5 min rinses at room temperature in phosphate-buffered saline plus 0.1% (v/v) Tween-20, ECL detection was carried out using the SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL) according to the manufacturer's instructions. Blots were exposed to X-ray film, and signal intensities were quantitated using the Alpha Imager 1D-Multi function (Alpha Innotech).

Nuclear Extract Preparation

Three to four confluent T150 flasks were used for each treatment and nuclear extract preparation. Prior to treatment, T47D cells were serum-starved for 24 h. T47D and HMEC cells were untreated or treated for 15 min with 200 U/ml IFN- γ \pm 25 ng/ml TNF- α . Nuclear extracts were prepared as described by Dignam et al., [1983] with minor modifications. The following protease and phosphatase inhibitors, along with 1 mM DTT, were added to the PBS/5 mM EDTA (used for detaching cells), hypotonic buffer, and nuclear extraction buffer just before use: 1 mM Na₃VO₄, 1 mM NaF, 1 mM Na₂MoO₄, 5 μ g/ml aprotinin, 1 μ g/ml pepstatin A, and 1 mM PMSF. Cellular lysis was achieved using final concentrations of 0.1% or 0.5% (v/v) NP-40 for T47D cells and HMEC, respectively. Nuclear extracts were collected and dialyzed in Slide-A-Lyzer MINI dialysis units (Pierce) for 4 h against 250 ml dialysis buffer, changing buffer once. Dialyzed nuclear extracts were flash-frozen in dry ice/ethanol and stored in usable aliquots at -80°C. Protein concentration was determined by the method of Lowry et al., [1951]. Nuclear extracts of at least 5 mg protein/ml concentration were used for EMSA.

Electrophoretic Mobility Shift Assay (EMSA) and Supershift Assay

EMSA and supershift assays for analysis of the MUC1 promoter STAT-binding element at -503/-495 were carried out using the STAT1 α NuShift kit (Geneka Biotechnology, Montreal, Canada) according to the manufacturer's instructions. Oligonucleotide probes for STAT1 α EMSA included the MUC1 promoter potential STAT-binding site flanked by additional native sequence (-513/-485; 5'GGTGGGGCTA[TTCCGGGAA]GTGGTGGGGG3'; Sigma-Genosys) and a positive control STAT-inducible element (SIE) (5'GTCGACAT-[TTCCCGTAA]ATCGTCGA3'; provided with kit). Corresponding mutant oligos used in competition assays included a mutant MUC1 potential STAT-binding site (5'GGTGGGGCTA[ccCGGGAA]GTGGTGGGGG3') and a provided mutant SIE (5'GTCGACAT-[aTagCGTAA]ATCGTCGA3') (mutated nucleotides in lowercase). Double-stranded oligos were prepared by combining equal parts sense and antisense oligos, boiling for 5 min, and cooling to room temperature overnight to anneal. Wild-type oligos were end-labeled using $\gamma^{32}\text{P}$ -ATP (NEN Life Science Products, Boston, MA) and T4 polynucleotide kinase (Roche). Unlabeled wild-type and mutant competitor oligos were used in a 100-fold molar excess over labeled probe. Five micrograms of T47D or HMEC nuclear extract protein were used in each reaction. Nuclear extracts from IFN- α -treated U-937 cells were provided as a positive control. For supershift assays, nuclear extracts were preincubated with NRS (Calbiochem-Behring) or rabbit polyclonal STAT1 α antibody (Geneka Biotechnology). EMSA analysis of the MUC1 promoter potential κB site at -589/-580 was carried out using the NF κB /Rel family NuShift kit (Geneka Biotechnology) according to the manufacturer's instructions. Oligonucleotide probes and competitors for NF κB /Rel EMSA included the MUC1 promoter potential κB site flanked by additional native sequence (MUC κB ; -599/-570; 5'-CCAGGCTGCT[GGAAAGTCCG]GCTGGGGCGG-3'); the corresponding mutant, mMUC κB (5'-CCAGGCTGCT[GGcccGTCCG]GCTGGGGCGG-3'; Sigma-Genosys); a provided control binding site for NF κB p65 and c-Rel, Rel (5'-AGCTT[GGGGTATTTCC]AGCCG-3'); the corresponding mutant, mRel (5'-AGCTT[GGcaTAggTCC]AGCCG-3'); a provided control binding site for

NF κB p50, NF κB (5'-GCCATGG[GGGGATCCC]CGAAGTCC-3'); and the corresponding mutant, mNF κB (5'-GCCATGG[GccGATCCC]CGAAGTCC-3'). For supershift assays, nuclear extracts were preincubated with NRS or a rabbit polyclonal antibody to c-Rel, NF κB p65, or NF κB p50 (Geneka Biotechnology).

RESULTS

TNF- α Greatly Enhances MUC1 Transcriptional Activity in the Presence of IFN- γ

A segment of 5' flanking sequence of the human MUC1 gene from -1,406 to +33 was used in transient transfection assays to study responsiveness to IFN- γ , IL-1 β , or TNF- α in T47D cells. A modest but significant stimulation of reporter activity (2.5- to 5-fold) was observed in cells treated independently with IL-1 β , TNF- α , or IFN- γ compared with untreated cells (Fig. 1). Reporter activity of cells treated with IL-1 β and TNF- α in combination was not significantly different from that in cells treated with each cytokine singly, although combined treatment with IL-1 β and IFN- γ resulted in additive stimulation of MUC1 promoter activity. Most notably, a robust stimulation of MUC1 promoter activity (>15-fold) was observed in cells treated with TNF- α in the presence of IFN- γ . The apparent synergy of TNF- α and IFN- γ upon MUC1 promoter activity was not significantly enhanced by IL-1 β . Similar treatments had no effect on reporter activity of an unrelated promoter (for the thymidine kinase (TK) gene) in the same vector (data not shown). In transient transfections of T47D cells with the 1.4MUC promoter treated with various concentrations of either TNF- α or IFN- γ , maximal stimulation of MUC1 transcriptional activity was observed in the presence of 25 ng/ml TNF- α and 200 U/ml IFN- γ (data not shown). MUC1 promoter responsiveness to TNF- α and IFN- γ , singly and in combination, was observed within 3 h of treatment, reaching a peak at 24 h (data not shown). For these reasons, 25 ng/ml TNF- α and 200 U/ml IFN- γ were used to treat T47D cells for 24 h in all subsequent transient transfections for promoter construct evaluation. Northern blot analysis was performed on RNA from T47D cells to determine whether the changes in promoter activity translated into increases in steady-state levels of MUC1 mRNA. Two differently sized transcripts were observed as a result of allelic polymorphism in the VNTR

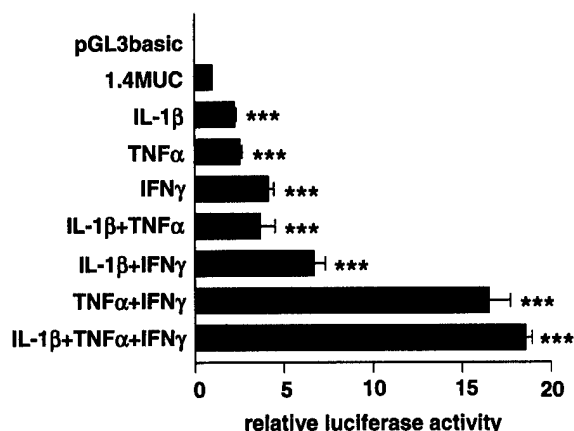


Fig. 1. MUC1 promoter response to proinflammatory cytokines. T47D cells were transiently transfected with control plasmid (pGL3basic) or 1.4 kb MUC1-luciferase (1.4MUC). Cells transfected with 1.4MUC were treated with the indicated cytokines for 24 h (IL-1 β , 10 ng/ml; TNF- α , 25 ng/ml; IFN- γ , 200 U/ml). Promoter activity was determined as described in Methods and is expressed as the ratio of firefly luciferase activity (pGL3 plasmids) to the activity of the co-transfected internal control, *Renilla* luciferase. Error bars reflect results of at least two experiments performed in duplicate \pm SEM. *** P < 0.001 versus 1.4MUC in untreated cells by ANOVA and Tukey-Kramer multiple comparisons test.

domain (Fig. 2A). Treatment of the cells with TNF- α alone did not noticeably affect the steady-state level of MUC1 mRNA from either allele, and IFN- γ alone resulted in a mild (approximately 50%) increase in the steady-state level of MUC1 mRNA (Fig. 2B). Combined treatment with TNF- α and IFN- γ increased the level of MUC1 mRNA by 2- to 2.5-fold, a significantly greater increase than with IFN- γ alone but less than predicted by reporter activity in transfected cells. Despite these apparent changes in MUC1 mRNA in response to TNF- α and IFN- γ , similar changes in the level of MUC1 protein were not evident (data not shown).

The high basal level of MUC1 expressed in T47D cells, along with variability in MUC1 promoter responsiveness to IFN- γ in other epithelial cancer cell lines (data not shown), prompted us to examine cytokine responsiveness in normal breast epithelial cells. HMEC, isolated from normal breast tissue and having a finite life span in culture, were treated with TNF- α and IFN- γ , independently and in combination for up to 48 h. Northern blot analysis of HMEC total RNA indicated that MUC1 mRNA was not detectable in either untreated cells or cells treated with TNF- α alone (Fig. 3A). However, transcription of MUC1 was induced by IFN- γ

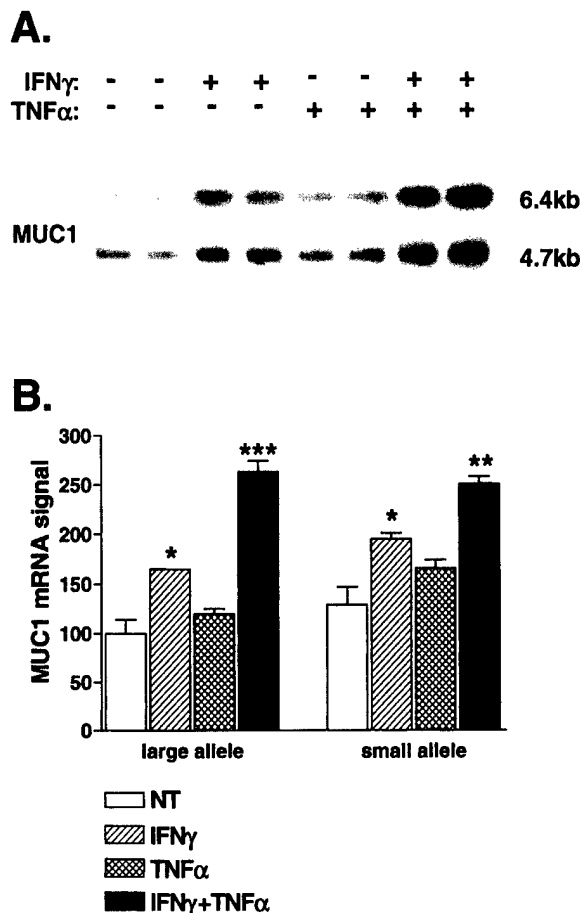


Fig. 2. Northern blot analysis of MUC1 mRNA in T47D cells cultured in the presence or absence of IFN- γ and/or TNF- α . Following a 24 h serum starvation, T47D cells were treated with 200 U/ml IFN- γ and/or 25 ng/ml TNF- α in the presence of 5% (v/v) serum for 24 h. (A) Total RNA was extracted and analyzed (6 μ g/lane) for MUC1 mRNA as described in Methods. (B) Signal intensities were quantified using the 1D-Multi function of an Alpha Imager and normalized to that of the larger MUC1 transcript in untreated cells. * P < 0.05, ** P < 0.01, *** P < 0.001 versus signal of transcript in non-treated (NT) cells.

alone, and this induction of expression was further enhanced by TNF- α . The effects of these treatments on MUC1 expression were observed within 12 h (data not shown). Comparison of the combined signals of both transcripts indicates that the apparent TNF- α -induced enhancement of the steady-state level of MUC1 mRNA in IFN- γ -treated cells is twofold, although when compared to untreated cells, the overall increase in MUC1 mRNA is essentially infinite. Separate analyses revealed that the level of the larger, less abundant transcript in IFN- γ -treated cells was increased fourfold by TNF- α , as compared to a 50% increase in the smaller transcript (Fig. 3B). Western blot analysis of HMEC total

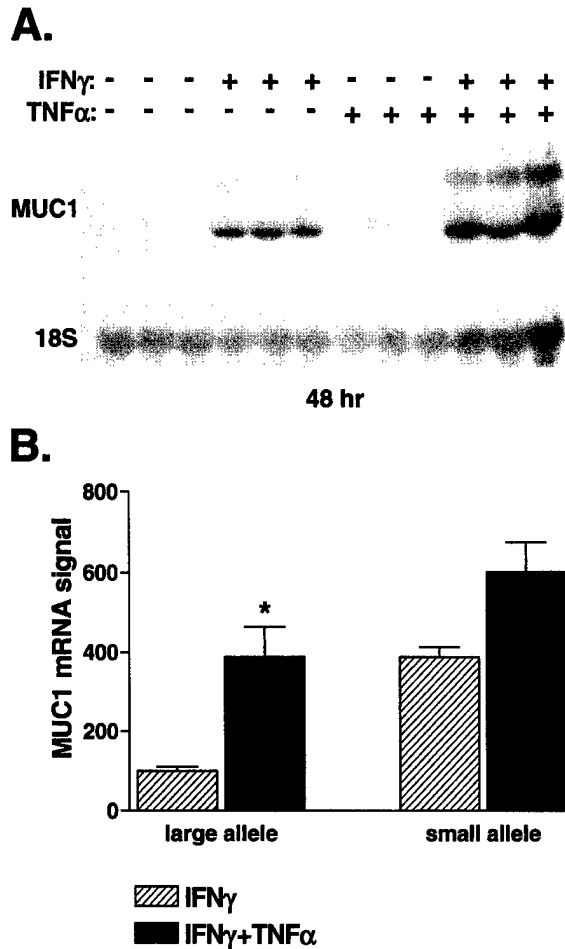


Fig. 3. MUC1 mRNA in HMEC cultured in the presence of IFN- γ and/or TNF- α . HMEC were treated with 200 U/ml IFN- γ and/or 50 ng/ml TNF- α for the indicated times. (A) Total RNA was extracted and analyzed (10 μ g/lane) for MUC1 mRNA or 18S ribosomal RNA as described in Methods. (B) Signal intensities were quantified using the 1D-Multi function of an Alpha Imager and normalized to the larger MUC1 transcript in IFN- γ -treated cells. * P = 0.0200 versus signal of transcript in IFN- γ -treated cells by unpaired t -test.

cellular protein demonstrated induction of MUC1 expression in response to IFN- γ alone that was further enhanced by co-treatment of the cells with TNF- α (Fig. 4A). MUC1 protein levels were increased by TNF- α at least 2.5-fold over that observed in cells treated with IFN- γ alone (Fig. 4B). After prolonged exposure, induction of MUC1 by TNF- α alone was also detectable (data not shown).

The Mechanism of IFN- γ Stimulation of MUC1 Expression is Conserved Between Breast Cancer Cells and Normal Mammary Epithelia

We determined that the *cis*-acting element required for stimulation of the MUC1 promoter

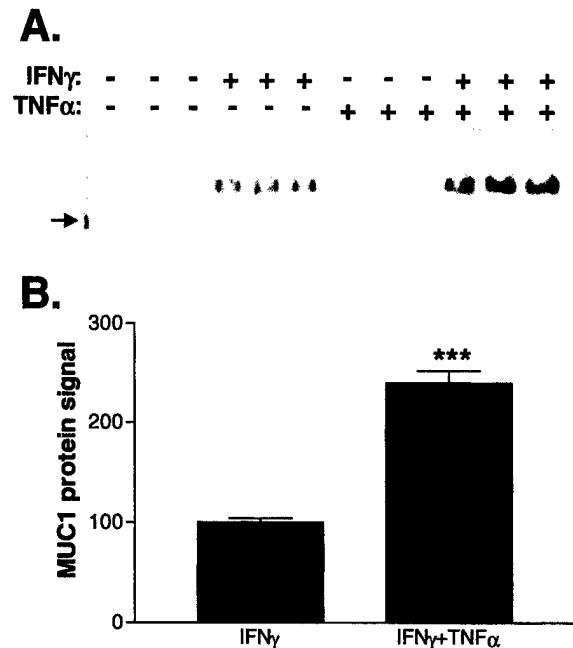


Fig. 4. Western blot analysis of MUC1 in HMEC cultured in the presence of IFN- γ and/or TNF- α . HMEC were treated with 200 U/ml IFN- γ and/or 50 ng/ml TNF- α for 48 h. (A) Total cellular protein (50 μ g/lane) was analyzed for changes in MUC1 expression using the 214D4 antibody. The migration position of myosin (202 kDa) is indicated by the arrow. (B) Signal intensities of cell-associated MUC1 were quantified using the 1D-Multi function of an Alpha Imager and normalized to that in IFN- γ -treated cells. *** P = 0.0004 versus MUC1 signal in IFN- γ -treated cells by unpaired t -test.

by IFN- γ in T47D cells was the STAT-binding element at -503/-495, and this finding was independently confirmed [Lagow and Carson, 1999; Gaemers et al., 2001]. However, IFN- γ responsiveness of MUC1 at the level of transcript differed greatly between T47D cells and HMEC. For this reason, we examined HMEC to determine whether STAT1 α also mediated induction of MUC1 expression by IFN- γ in normal breast epithelial cells using electrophoretic mobility shift assays (Fig. 5). A 29-bp double-stranded oligonucleotide encompassing the STAT-binding element from the MUC1 promoter (-513/-485) and a control SIE oligonucleotide were used to probe nuclear extracts prepared from untreated HMEC and T47D cells or cells treated with 200 U/ml IFN- γ for 15 min. No complexes were observed with nuclear extracts from untreated HMEC or T47D cells (lanes 1 and 7); however, incubation of the MUC1 or SIE probe with extracts from IFN- γ -treated cells or control extracts resulted in distinct protein-DNA complexes that migrated

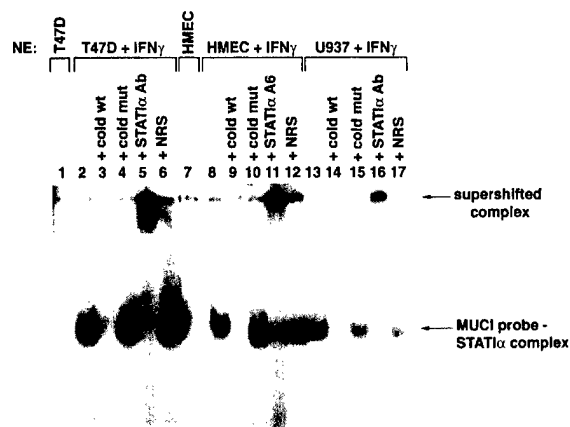


Fig. 5. Electrophoretic mobility shift assay of the MUC1 promoter STAT-binding element and nuclear extracts from T47D and HMEC cells. A 29-bp end-labeled fragment of the MUC1 promoter (–513/–485) or a control STAT-inducible element (SIE) was incubated with 5 μ g of T47D or HMEC nuclear protein. The SIE probe was also incubated with positive control nuclear protein from IFN- α -treated U937 cells. Unlabeled wild-type (wt) or mutated (mut) fragments were added at a 100-fold molar excess compared to labeled probes. For supershift analysis, rabbit polyclonal anti-STAT1 α or normal rabbit serum (NRS) was preincubated with nuclear proteins before addition of probe (1×10^5 cpm/reaction).

similarly (lanes 2, 8, and 13). Inclusion of excess unlabeled wild-type oligonucleotide in the binding reaction completely prevented complex formation, demonstrating specificity of the bound factor(s) for the STAT-binding element (lanes 3 and 9). Unlabeled mutant oligonucleotide did not affect complex formation (lanes 4 and 10). Pre-incubation of nuclear extracts with a rabbit polyclonal STAT1 α antibody, but not NRS, further retarded the mobility of the complex observed with extracts from IFN- γ -treated cells, demonstrating that STAT1 α was a component of the complex in the cancer cell line and in normal mammary epithelia (lanes 5, 6, 11, and 12).

Transcriptional Synergy by IFN- γ and TNF- α Requires the κ B Site at –589/–580 and the STAT-Binding Site at –503/–495 in the MUC1 Promoter

We assessed the requirement of *cis*-acting elements in the MUC1 promoter for mediating synergistic responses to IFN- γ and TNF- α , initially employing deletion analyses and transient transfection assays (Fig. 6). The –604/+33 construct retained the full synergistic response to IFN- γ and TNF- α , indicating that the *cis*-acting elements necessary for this response were present. Deletion to –570, excluding a

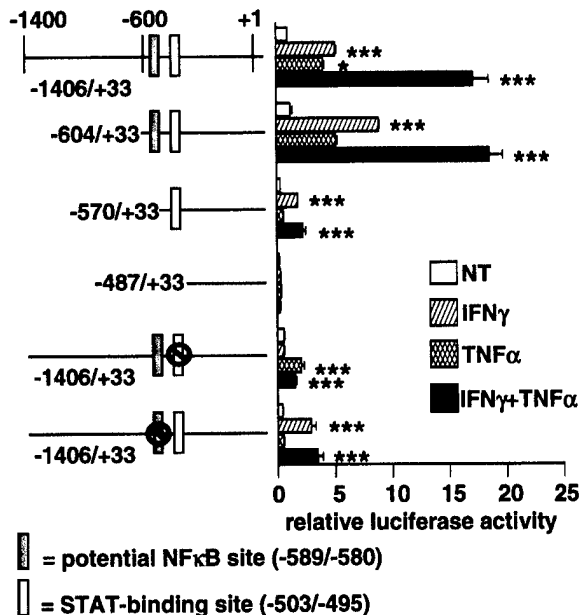


Fig. 6. Synergistic response of the MUC1 promoter to IFN- γ and TNF- α requires a κ B site at –589/–580 and the STAT-binding site at –503/–495. T47D cells were transiently transfected with the full-length MUC1 promoter construct, 1.4MUC, or the indicated mutant constructs and subsequently treated with 200 U/ml IFN- γ and/or 25 ng/ml TNF- α for 24 h. All promoter activities were normalized to that of 1.4MUC in untreated cells. Error bars reflect results of at least two experiments performed in triplicate \pm SEM. * P < 0.05, *** P < 0.001 vs. activity in non-treated (NT) cells by ANOVA and Tukey–Kramer multiple comparisons test.

potential κ B site at –589/–580, but retaining the STAT-binding site at –503/–495, reduced basal promoter activity by 60%. The response of the –570 construct to IFN- γ was approximately fivefold over that in untreated cells; however, TNF- α responsiveness was eliminated. Additionally, the magnitude of the response to IFN- γ in the presence of TNF- α was not significantly greater than with IFN- γ alone. Further deletion to –487/+33 eliminated both TNF- α and IFN- γ responsiveness as well as 75–80% of the basal promoter activity. Specific mutation of the STAT-binding element at –503/–495 abolished IFN- γ responsiveness and prevented IFN- γ /TNF- α synergy; however, a three to fourfold response to TNF- α alone was retained, similar in degree to that observed with the intact 1.4 kb promoter. Likewise, specific mutation of the κ B site at –589/–580 eliminated TNF- α responsiveness, but retained a five to sixfold response to IFN- γ , and promoter activity in the presence of both TNF- α and IFN- γ was not greater than with IFN- γ alone. Furthermore, specific mutation of either the STAT site or the κ B site reduced basal MUC1 promoter activity by 45–50%.

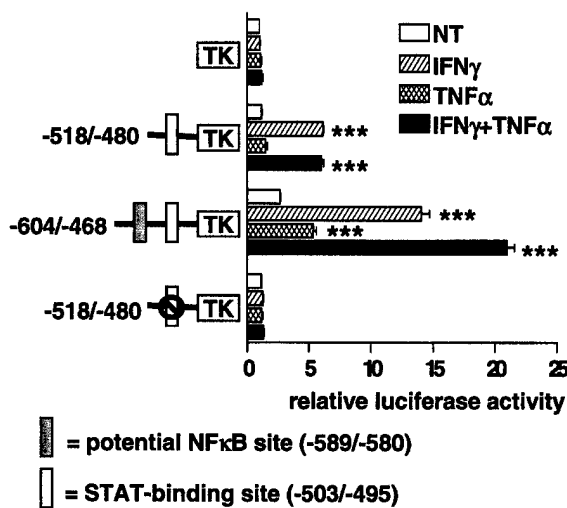


Fig. 7. The MUC1 promoter STAT-binding element is necessary but not sufficient for transcriptional synergy by IFN- γ and TNF- α . Fragments of the MUC1 promoter (-518/-480) containing a wild-type (TTCCGGGAA) or mutated (ccCCGGGAA) STAT-binding element, and a fragment containing both the STAT-binding element and a potential κ B site (-604/-468), upstream of the TK promoter in pGL3basic were transfected into T47D cells treated with 200 U/ml IFN- γ and/or 25 ng/ml TNF- α for 24 h. Construct activities were normalized to that of the vector, pGL3-TK, in untreated cells. Error bars reflect results of at least two experiments performed in triplicate \pm SEM. *** P < 0.001 versus activity in non-treated (NT) cells by ANOVA and Tukey-Kramer multiple comparisons test.

Heterologous promoters containing a wild-type or mutant STAT-binding element (-518/-480) upstream of the TK promoter were also used in transient transfections of T47D cells to assess the response to TNF- α and IFN- γ (Fig. 7).

The construct containing a wild-type STAT-binding element displayed no responsiveness to TNF- α alone, and in cells treated with both IFN- γ and TNF- α , the promoter activity was not significantly greater than with IFN- γ alone. Inclusion of additional 5' sequence in the -604/-468 construct, containing both the potential κ B site at -589/-580 and the STAT-binding site at -503/-495, not only conferred responsiveness to TNF- α alone upon the TK promoter, but also a synergistic response to TNF- α and IFN- γ . As expected, specific mutation of the STAT-binding site completely abolished responsiveness to IFN- γ , and activity of this construct was not affected by TNF- α .

TNF- α Induces Binding of NF κ B to the MUC1 Promoter κ B Site

We assessed function of the putative κ B site in TNF- α regulation of MUC1 transcription using electrophoretic mobility shift assays. MUC1 promoter sequence from -599 to -570 was used to probe nuclear extracts from T47D cells that were treated for 15 min with 25 ng/ml TNF- α in the presence or absence of 200 U/ml IFN- γ (Fig. 8). Several complexes of different mobilities were observed and were numbered in order of slowest to fastest mobility. Complex 1 (C1) was observed with all T47D nuclear extracts tested, regardless of treatment; however, C2 and C3 were specifically formed with nuclear extracts from T47D cells that had been treated with TNF- α (Fig. 8A; lanes 3 and 4). The

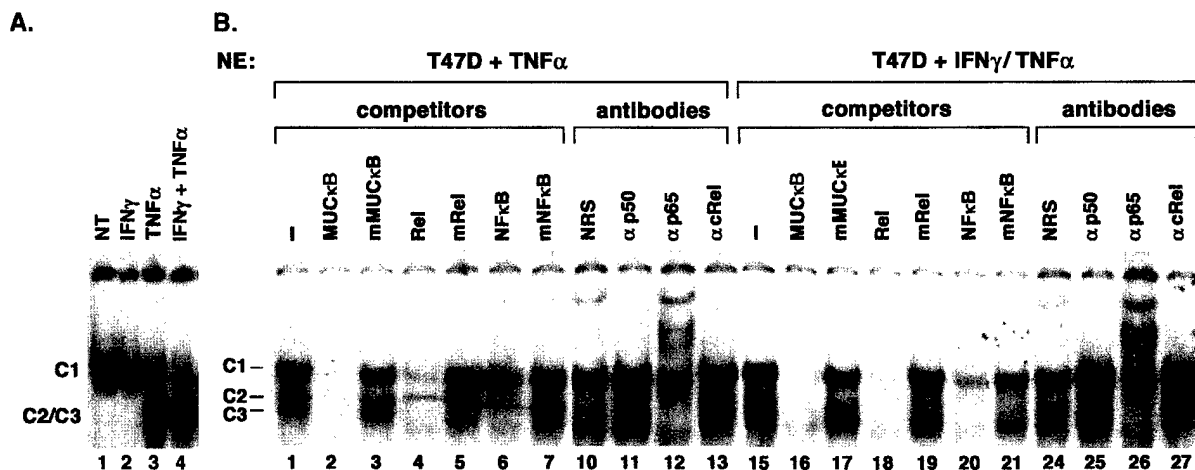


Fig. 8. Electrophoretic mobility shift assay of the MUC1 promoter potential κ B site and nuclear extracts from T47D cells. (A) A 30-bp end-labeled fragment of the MUC1 promoter (-599/-570) was incubated with 5 μ g of nuclear extract protein from T47D cells treated with or without 25 ng/ml TNF- α \pm 200 U/ml IFN- γ for 15 min. (B) Unlabeled wild-type or mutant (m) oligo-

nucleotides were added at a 100-fold molar excess compared to labeled probe in competition assays as described in Methods. For supershift analysis, nuclear extracts were preincubated with NRS or rabbit polyclonal antibodies to NF κ B p50, NF κ B p65, or c-Rel for 20 min before addition of probe ($> 1 \times 10^5$ cpm/reaction). Observed complexes were labeled as C1-C3.

intensities of C2 and C3 were similar in reactions containing nuclear extracts from cells treated with TNF- α in the presence or absence of IFN- γ , indicating that the bound factor(s) were not induced to bind differently upon the addition of IFN- γ . In competition assays, excess unlabeled MUC κ B, but not a corresponding mutant sequence, mMUC κ B, completely abolished C1, C2, and C3, although a very faint band was still observed migrating slightly faster than C3 (Fig. 8B, lanes 2, 3, 16, and 17). Excess unlabeled Rel control oligonucleotide (a binding site for c-Rel and NF κ B p65), but not a corresponding mutated sequence, competed similarly (lanes 4, 5, 18, and 19); however, neither excess unlabeled NF κ B control (a binding site for NF κ B p50) nor its mutated control, competed for binding (lanes 6, 7, 20, and 21).

Supershift assays were performed with a series of antibodies specific for several NF κ B/Rel family members to determine the identity of the bound factor(s). Preincubation of nuclear extracts with NRS did not affect the mobility of any of the complexes, but did yield a nonspecific band of slower mobility than C1 (Fig. 8B, lanes 10 and 24). Antibodies for NF κ B p50 (NF κ B1) and c-Rel did not retard the mobility of any complex (lanes 11, 13, 25, and 27); however, in addition to generating the nonspecific band observed with NRS, antiserum for NF κ B p65 (RelA) partially shifted C1 and completely shifted C2 and C3, identifying p65 as a component of these complexes (lanes 12 and 26).

DISCUSSION

Studies of MUC1 transcriptional regulation so far have relied heavily on the use of transformed cell lines. Breast and pancreatic cancer cell lines in which MUC1 promoter analyses have been conducted overexpress MUC1, indicating gene regulation gone awry. While MUC1 promoter studies in cancer cell lines are informative, comparison with normal, untransformed cells are needed to provide insight as to where regulatory aberrations may occur. We have demonstrated that MUC1 expression is amplified by TNF- α in the presence of IFN- γ , modestly in T47D breast cancer cells and robustly in normal HMEC. At the transcriptional level, this regulation requires the independent actions of STAT1 α and NF κ B on their respective binding sites at -503/-495 and -589/-580 in the MUC1 promoter. Given that the binding of NF κ B to the

MUC1 κ B site was not enhanced by the addition of IFN- γ to the TNF- α treatment, the transcriptional synergy appears not to be mediated by cooperative binding of these transcription factors. In addition, the MUC1 STAT-binding site did not compete out any complexes formed by the MUC1 κ B site incubated with nuclear extracts from IFN- γ /TNF- α -treated cells, and an antibody for STAT1 α did not affect any of the complexes (data not shown), demonstrating that the synergy is mediated by the independent actions of STAT1 α and NF κ B on their respective sites in the MUC1 promoter and subsequent cooperative action of these factors upon basal transcription elements.

We found that the steady-state levels of MUC1 mRNA in T47D cells treated with IFN- γ in the presence or absence of TNF- α did not reflect promoter activity as measured in transient transfection assays. Several factors might potentially account for these discrepancies. Despite the fact that overexpression of MUC1 in mammary tumors was observed using the same 1.4 kb MUC1 promoter [Graham et al., 2001], additional regulatory elements present in the endogenous MUC1 gene may account for the high basal level of MUC1 expressed by T47D cells. Therefore, activity of the 1.4 kb MUC1 promoter in T47D cells may be considerably less than that of the endogenous promoter so that the basal level of promoter activity appears to be lower, allowing for greater apparent stimulation by proinflammatory cytokines. Conversely, further stimulation of MUC1 expression may be dependent on the basal level of transcription in the cell line being studied. In this regard, T47D cells have been reported to express very high basal levels of MUC1 relative to other breast cancer cell lines examined [Walsh et al., 1999].

In contrast to the modest cytokine responsiveness of MUC1 in T47D cells, robust induction of expression was observed in the normal mammary epithelial cells treated with IFN- γ alone or in combination with TNF- α , resulting from transcriptional stimulation. However, we noted that the degree of MUC1 induction in HMEC by IFN- γ alone appeared to increase with increasing passage number of the cells. In earlier passage HMEC, the difference in MUC1 protein level in cells treated with IFN- γ alone versus IFN- γ and TNF- α was more pronounced relative to the difference in the level of transcript, suggesting the potential involvement of post-transcriptional mechanisms in addition to

transcriptional mechanisms in normal cells. Several mechanisms for IFN- γ /TNF- α synergy have been reported, including cross-regulation of receptors, enhancement of STAT1 activity by TNF- α , cooperation between STAT1 α or IRF-1 and NF κ B, and IFN- γ augmentation of NF κ B activation via rapid I κ B degradation [Sanceau et al., 1995; Cheshire and Baldwin, 1997; Ohmori et al., 1997; Lee et al., 2000]. Augmentation of NF κ B or STAT binding falls within the realm of transcriptional control and would be most likely be detectable in gel shift assays. MUC1 mRNA was detectable in HMEC after 12 h of treatment with IFN- γ alone or in combination with TNF- α , and although earlier timepoints were not examined in these cells, increased promoter activity was observed within 3 h of treatment in T47D cells. In this respect, synthesis of additional factors that may participate in the regulation is unlikely. The discrepancy between the level of MUC1 protein and mRNA observed in early passage HMEC treated with TNF- α and IFN- γ might be explained by increased protein stability as a result of increased translation or decreased degradation. Factors potentially affecting the rate of degradation may include post-translational modification, such as phosphorylation or glycosylation, or association with other proteins. Treatment of primary cultures of rat mammary epithelial cells or a rat mammary carcinoma cell line, 13,762, with TGF β 1 results in differences in post-transcriptional regulation of another transmembrane mucin, SMC/Muc4 [Price-Schiavi et al., 1998]. Thus, post-transcriptional regulation may modulate expression of multiple mucins.

Glycosylation of MUC1 also is regulated and can vary within a tissue [Aplin et al., 1998; Hanisch and Muller, 2000]. Whether this post-translational modification of MUC1 is affected by the cytokine treatments described has not yet been addressed. The apparent difference in MUC1 protein levels in HMEC treated with IFN- γ alone versus IFN- γ and TNF- α might reflect differential recognition by the 214D4 antibody; however, this would be true only if a particular treatment induced a glycoform switch, i.e., IFN- γ and TNF- α treatment together producing an underglycosylated, more recognizable, form of MUC1 in contrast to treatment with IFN- γ alone. Comparison of 214D4 with other antibodies directed toward the MUC1 tandem repeats [Xing et al., 1989],

DF3 [Perey et al., 1992], HMFG1, HMFG2 [Taylor-Papadimitriou et al., 1981; Burchell et al., 1983], and SM3 [Burchell et al., 1987] indicated that unlike most, 214D4 appears to recognize MUC1 protein independently of glycosylation state (J. Julian, unpublished observations).

At first glance, the STAT-binding element at -503/-495 appeared to be important for constitutive as well as stimulated MUC1 expression, as specific mutation of this element reduced promoter activity by 40–50% in T47D cells [Lagow and Carson, 1999; Gaemers et al., 2001]. Nonetheless, the STAT element did not bind components of nuclear extracts from unstimulated T47D cells in gel shift assays. Furthermore, unpublished studies conducted in our lab demonstrated that MUC1 expression was unchanged by overexpression of STAT1 α in T47D cells, indicating that the high basal level of MUC1 expression in T47D cells was not accounted for by the action of this factor upon the STAT-binding element. Conversely, the κ B element may be involved in constitutive activation of the MUC1 promoter. Regardless of treatment, a prominent complex was observed in gel shift assays of the MUC1 κ B site and nuclear extracts from T47D cells. Constitutively active NF κ B has been reported in some non-epithelial, normal cell types [May and Ghosh, 1997], although NF κ B DNA-binding activity is more often induced. Studies of the MUC1 κ B site in normal mammary epithelial cells are currently underway to determine whether constitutive activation of this element is a normal feature of MUC1 transcriptional regulation. When analyzing nuclear extracts from T47D cells, the constitutive complex of slowest mobility (C1) was specifically competed by excess unlabeled wild-type but not the mutated MUC1 κ B site, indicating binding specificity. An antibody to NF κ B p65 partially shifted complex C1 in addition to the complete shift of induced complexes C2 and C3, indicating that p65 may participate in both basal and TNF- γ -stimulated MUC1 transcription. Besides forming homodimers, NF κ B p65 has been shown to form heterodimers with c-Rel and p50 [May and Ghosh, 1997]; however, supershift assays did not indicate involvement of these factors in the constitutive or TNF- α -induced complexes. As C1 was not an induced complex, additional assays with antibodies for other NF κ B/Rel family members were not performed.

Overexpression of MUC1 is a common characteristic of many human epithelial-derived cancers including those of the breast, ovary, and pancreas, and malignant transformation is associated with abnormal regulation of MUC1 expression at multiple levels [Abe and Kufe, 1990; Gendler et al., 1990; Bieche and Lidereau, 1997; Waltz et al., 1998]. In contrast to the apically-restricted, highly glycosylated molecule observed in normal luminal epithelial cells, MUC1 in tumor cells is expressed over the entire plasma membrane [Hilkens et al., 1992], accumulates in the cytoplasm [Peterson et al., 1990; Rahn et al., 2001], and is drastically underglycosylated and antigenically distinct in tumor cells [Girling et al., 1989], so that humoral and cell-mediated immune responses are elicited; however, these responses are often insufficient to destroy the tumor cells [Jerome et al., 1991; van de Wiel-van Kemenade et al., 1993; Kotera et al., 1994]. The demonstration that MUC1 expressed by cancer cells inhibits T-cell proliferation or activation may provide a mechanistic explanation for the ineffectiveness of the immune response [Gimmi et al., 1996; Agrawal et al., 1998b; Chan et al., 1999, 2000]. In this context, persistent cytokine stimulation of breast tumor cells may result in a positive feedback loop promoting tumor cell survival through overexpression of MUC1.

In conclusion, we report that TNF- α amplifies MUC1 expression in IFN- γ -treated breast cancer cells and to a much greater degree in normal mammary epithelial cells. At the transcriptional level, we show that this concerted regulation requires the independent actions of NF- κ B p65 and STAT1 α upon their respective binding sites at -589/-580 and -503/-495 in the MUC1 promoter. Additionally, our findings indicate that post-transcriptional mechanisms also may be involved in TNF- α /IFN- γ regulation of MUC1 expression in the normal cells. The discrepancy between MUC1 promoter activity and expression in T47D cells treated with IFN- γ in the presence or absence of TNF- α suggests that elements outside of the 1.4 kb 5' flanking region override these normal controls in tumor cells.

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